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The aim of this project is to understand the mechanism of transition of breast cancer from hormone-dependent state to hormone-independent state. In order to pursue ths question we have designed a regulatable regulator that can recognize all estrogen receptor target genes and put them under the regulation of exogenous compound RU486. In the presence of RU486, this regulator can bind to all estrogen targets and shut down their expression. We want to use this regulator to study the role of estrogen receptor target genes on breast cancer cell growth in hormone-dependent and hormone-independent states. In the last year, using a cellular culture system, we have successfully constructed this regulator and demonstrated that it can shut down a estrogen receptor reporter gene. With the success of this regulator, now we can answer what we set out to do in the next grant period.

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FOREWORD

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Jimmy Tsai 7/3/97

PI - Signature Date

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Introduction

Breast cancer is the most frequent cancer in American women (1). Despite extensive studies undertaken to understand the etiology of breast cancer, no clear picture has emerged. It is thought that genetic, dietary, hormonal, environmental and lifestyle factors contribute to the incidence of this disease (1). In contrast to many other cancers, the incidence of breast cancer has steadily increased with a 24% increase between 1973 and 1987 (1). Thus, breast cancer remains a major problem to overcome in the improvement of women's health in America.

Previous attempts to identify genes responsible for breast cancer have identified several oncogenes, such as wnt-1 (int-1) , wnt-3 and int-2, whose increased expression is frequently observed in MMTV-induced tumors(2). Furthermore, expression of these genes in mammary epithelial cells enables them to continue to grow even in a dense culture (3-5). These results provide strong evidence for their involvement in tumor development. The oncogenic potential of these genes has been demonstrated in transgenic animals. In addition, mutation of several tumor suppressor genes has also been shown to correlate with breast cancer (6,7).

Steroid hormones, estrogen and progesterone, and their receptors have also been demonstrated to be associated with breast cancer. Breast cell proliferated in response to estrogen and progesterone increases this proliferation potential. In addition, breast tumors rarely develop in ovariectomized woman (8). Furthermore, some women with breast cancer have higher estrogen levels than healthy control women (1) and antiestrogen treatment in breast cancer patients drastically reduces tumor reoccurrence (8,9). Finally, a strong correlation exists between reproductive history and the incidence of breast cancer (10).

It is well documented that initial breast cell growth and breast carcinoma is hormone-dependent. Antiestrogen treatment results in the arrest or remission of breast cancer growth (8,9). However, subsequently, most advance breast cancers become resistant to estrogen-ablation therapy (11). It has been proposed that mutation of the estrogen receptor (ER) to a constitutively active regulator or to a receptor which can be activated by estrogen antagonist, tomaxifen, may contribute to the transition from estrogen-dependent to -independent tumor growth [for review see (11)]. However, half of all advanced breast cancers are receptor positive but resistant to antiestrogen therapy and some ER negative tumors behave as if they are ER positive in expression of ER target genes such as progesterone receptor. Furthermore, many of ER mutations identified in tumor cells are also found in healthy cells of breast cancer patients or healthy individuals. thus, it remains controversial whether ER mutations have primary role in the transition from estrogen-dependent to -independent state.

In this proposal, we design experiments to dissect the role of ER target genes in the growth of breast cancer and to understand how transition from estrogen-dependent to-independent cancer growth occurs. We expect that results obtained from these studies will help us to devise a way to control breast tumor growth.

In the last year since we obtained the support for DAMD we have successfully constructed several regulators that can shut down the expression of ER dependent target reporter in a test transfection system. These results are discussed in detail in the next section.

A. Experimental Methods

(i) Plasmid construction

KRAB domain and E2F1 DNA binding domain were amplified from pBXG1/Kid-1N and pCMV-E2F1 and ligated together by PCR. The resultant fragment was cut by Xba1 and EcoRI and inserted into MCS sites of pBS-KSII(+) to constructed pKS-KE. The truncated PR-LBD(-19)-KRAB fusion fragment was amplified from pCEP4/GLK by PCR. The PCR product was cut by DraI and EcoRI, terminal end fill-in by klenow, then blunt-end ligated into EcoRV site of pKS-KE. The resultant plasmid pKS-KEPE contains functional KRAB domain at both N- and C-terminals of chimeric construct. E2F1 BNA binding domain was cut out using BamHI and EcoRI and replaced by PCR amplified ER DNA-binding domain to generate regulatable repressor of ER target genes pKS-KEDPK. The plasmid pKS-KEDPK was then subcloned into pCMX expression vector and checked in frame and by sequence analysis and by *in vitro* transcription/translation. The primers used for PCR amplification (5' primer; 3' primer) are as follows:

KRAB: AAGCTTCTAGACTGCAGCTCGAGGCCACCATGGCTCCTGAGCAAAG;
CCGCTTCACGGGATCCTCTCCTTGCTG.

E2F1-DBD: GAGGATCCCGTCAAGCGGAGGCTGGAC;
CCGGAATTCGGAGATCTGAAAGTTCTC.

ER-DBD: CGCGGATCCTATGGAATCTGCCAAGGAG;
CGGAATTCAGACCCCACTTCACCCCTG.

PR-LBD &
KRAB fusion: CGCGGATCCTTTAAAAAGTTCAATAAAGTCAGAG;
CCGGAATTCTCATCCTTGCTGCAACAGGGAG.

(ii) Transfection

Hela cells were routinely maintained in Dulbeco's Modified Medium (DMEM; Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT). Cells were seeded 24 hours before transfection in 6-well tissue culture plates (2 x 10 5 cells per well) in phenol red-free DMEM contained 10% charcoal/dextran treated FCS. DNA was introduced into cells using lipofectin (Gibco, Gaithersburg, MD) following the technique instruction. Cells were transfected for 6 hours and then washed with phosphate buffer to remove the lipofectin. Cells were incubated for an additional 24 hours in phenol red-free medium containing 10% charcoal/dextran treated FCS with or without hormones, as indicated in the text. Cell extracts were prepared by adding 30µl lysis buffer (Promega, Madison, WI) and assayed for luciferase activities (Monolight 2010 Luminometer, Analytical Luminescence Laboratory, MI). All determinations were performed in quadruple in at least two independent experiments.

B. Results and Discussion

The plasmid pCMX-KEDPK expressing chimeric repressor of ER was constructed which contained a KRAB repressor domain at both N- and C-terminals, an ER DNA-binding domain and a truncated progesterone receptor ligand-binding domain. The construct was confirmed by sequence analysis. An expected size of the

chimeric protein was produced by *in vitro* transcription/translation (data not shown). The capacity of repressor KEDPK to block the ER mediated transcription was tested by co-transfecting plasmids carrying KEDPK, human ER and 3(ERE) tataLuc into HeLa cells (Figure 1). The luciferase activity was measured 24 hours following treatment with 17β-estradiol and Ru486. In the presence of Ru486 (10nM) the repressor KEDPK significantly inhibits the luciferase activity induced by ER. Fifty percent inhibition was observed when transfected equally amount of ER and repressor KEDPK. The inhibitory potency of KEDPK on ER transcription activity was shown in a dose-dependent manner (Figure 2). KEDPK did not interfere the transcription activity of ER in the absence of Ru486. The inhibitory activity of KEDPK was tightly regulated by Ru486, with maximal effect at concentration of 10 nM (Figure 3). Similar results were observed in breast cancer cell line MCF-7. The inhibitory activity of KEDPK was specific to the ER, since the repressor has shown no effect on other nuclear receptor systems tested so far in transient transfection (Figure 4).

Figure 1 INHIBITION OF ER ACTIVITY BY KEDPK

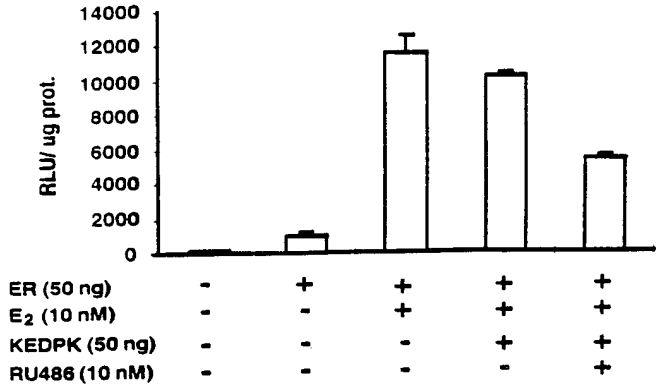


Figure 3 DOSE-DEPENDENT INHIBITION OF ER ACTIVITY

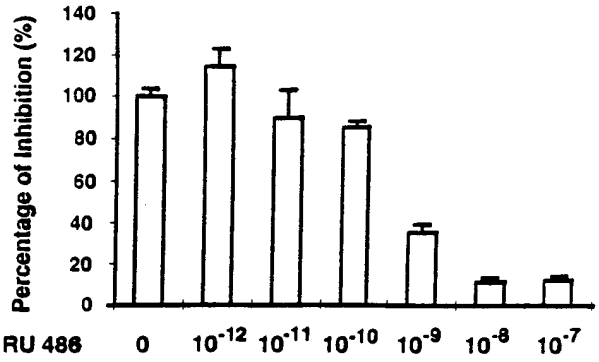


Figure 2 INIBITION OF ER ACTIVITY BY KEDPK IN DOSE-DEPENDENT MANNER

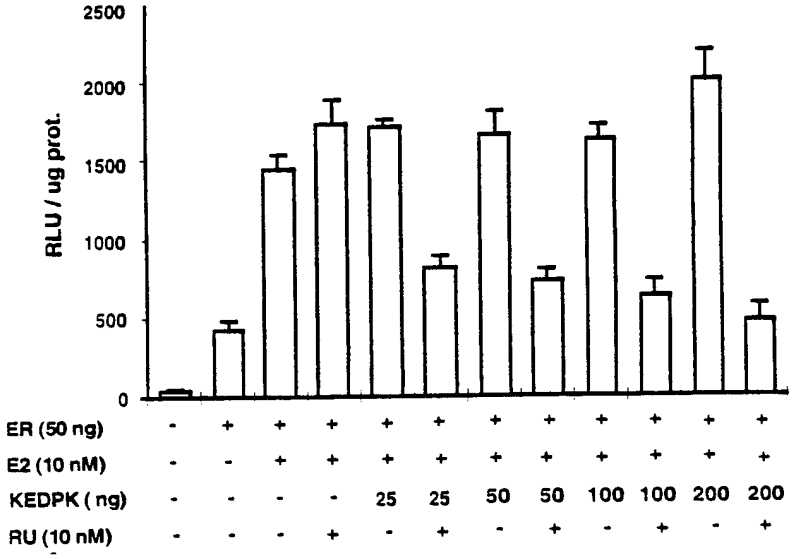
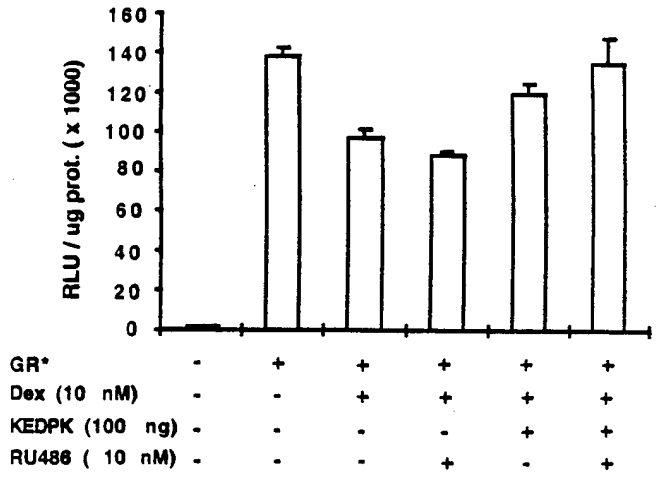


Figure 4 EFFECT OF KEDPK ON GR ACTIVITY



It is concluded that the chimeric repressor we designed could potentially and specifically inhibit ER target gene expression in response to exogenous ligand. This provided a useful tool to study the role of ER target genes breast cancer progression, and can be used as innovative strategies for gene therapy of breast cancer. Recently, we have successfully established cell lines from E_2-dependent MCF7 and E_2-independent MCF7-derived LTSD cells. Currently, we are analyzing the effectiveness of our regulator in shutting down ER target genes, P52, Myc and progesterone receptor, in a Ru486 dependent manner. These cell lines will then be used to study the role of ER target genes on transition of breast cancer cells from E_2-dependent to E_2-independent state.

Conclusions

In the last year, we have made major progress toward our goals to understand the transition of breast cancer cells from E2-dependent to E2-independent state. We have successfully constructed a regulator that can shut down ER target genes in a Ru486-dependent manner. We have tested it in a transfection system and it was shown to work well and specifically. We also successfully established stable cell lines from E2-dependent and -independent MCF7 cells. Its validity and effectiveness in controlling breast cancer cell growth will be tested in the coming year. We are optimistic that we will accomplish what we have proposed to do.

References

1. Henderson, B. E., Ross, R. K., and Pike, M. C. (1991) Science 254, 1131-1138.

2. Callahan, R.(1987) in The Mammary Gland Development, Regulation and Function. (Neville, M. C. and Daniel, C. Eds.), pp. 323-351, Plenum Publishing Inc. New York.

3. Brown, A. M. C., Wildin, R. S., Prendergast, T. J., and Varmus, H. E. (1986) Cell 46, 1001-1009.

4. Nusse, R. and Varmus, H. E. (1992) Cell 69, 1073-1087.

5. Rijsenijk, F., Van Deemter, L., Wagenaar, E., Sonnenberg, A., and Nusse, R. (1987) EMBO J. 6, 127-131.

6. Donehower, L. A., Godley, L. A., Aldaz, C. M., Pyle, R., Shi, Y.-P., Pinkel, D., Gray, J., Bradley, A., Medina, D., and Varmus, H. E. (1995) Genes and Dev. 9, 882-895.

7. Boyd, M., Harris, F., McFarlane, R., Davidson, H. R., and Black, D. M. (1995) Nature 375, 541-542.

8. Harris, J. R., Lippman, M. E., Veronesi, U., and Willett, W. (1992) N. Engl. J. Med. 327, 390-396.

9. Jodan, V. C. (1992) J. Natl. Cancer Inst. 84, 231-234.

10. Kelsy, J. L. and Gammon, M. D. (1991) Cancer J. Clin. 41, 146-165.

11. Horwitz, K. B.(1994) in Receptor-Mediated Biological Processes:Implications for Evaluating Carcinogenesis. pp. 29-45, Wiley-Liss,Inc.

Appendix

Abstract submitted to October meeting on Breast Cancer.

ROLE OF ESTROGEN RECEPTOR TARGET GENES IN BREAST CANCER

Dr. Zhi-Qing Ma, Dr. Sophia Y. Tsai and Dr. Ming-Jer Tsai

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Several lines of evidences indicate that breast cancer requires estrogen for initiation and maintenance until it progresses to a more aggressive stage. Two-third of estrogen receptor (ER) positive cancer patients initially response to anti-estrogen treatment and show tumor regression. Subsequently, most breast cancer cases cease to responsd to estrogen ablative therapy and progress into an aggressive hormone-independent state. It has been proposed that the ER plays an important role in both hormone-dependent and independent states. The specific aim of this project is to study the role of ER target genes in the promotion of breast cancer and the transit from an estrogen-dependent to an estrogen-independent state. The approaches we used is to construct a regulatable repressor of ER target genes by linking the DNA-binding domain of ER with Krupple-Associated Box (KRAB) repressor domain and a mutated progesterone ligand-binding domain which only responds to exogenous ligand. The regulatable repressor constructed in this way should bypass the alteration of signal transduction at ER level commonly occurred in breast cancer, including ER mutation, ligand-independent activation of ER. This repressor binds to ER responsive element (ERE) directly to turn off all the ER target genes in response to an exogenous ligand RU486. The construct was confirmed by sequencing and an expected size of the chimeric protein was produced by in vitro transcription/translation. The capacity of repressor to block the ER mediated transcription was tested by co-transfecting hER and an ERE containing reporter into Hela cells. In the presence of RU486 (10 nM) the repressor significantly inhibits the reporter activity induced by ER. The inhibitory potency on ER transcription activity was shown in a dose-dependent manner. The inhibitory activity of chimeric repressor was tightly regulated by RU486, with maximal effect at concentration of 10 nM. Similar results were observed in breast cancer cell line MCF-7. The inhibitory activity was specific to the ER, since the repressor has no effect on other nuclear receptor system tested.

Key words: Estrogen Receptor Target Gene, Regulatable Repressor, Tamoxifen Resistance, Breast Cancer

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD-17-96-1-6155

It is concluded that the chimeric repressor we designed could specifically inhibit ER target gene expression in response to exogenous ligand. This provided a useful tool to study the role of ER target genes in breast cancer progression, and can be used as innovative strategies for gene therapy of breast cancer.

ROLE OF ESTROGEN RECEPTOR TARGET GENES IN BREAST CANCER

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Expression of estrogen receptor (ER) plays an important role in breast tumorigenesis. Patients with ER positive tumor show the greatest benefits by treatment with anti-estrogen, tamoxifen. Nevertheless, many patients are insensitive to tamoxifen treatment, and eventually those patients who initially respond to tamoxifen become resistant. Many factors may contribute to these changes, including ER mutation and altered signal transduction that affect the activity of the receptor. The specific aim of this project is to further study the role of ER target genes in the promotion of breast cancer and design a vector to shut off its activity.

We have designed a chimeric regulator which can bind specifically to the estrogen response elememt and directly inhibit estrogen receptor target genes in the presence of exogenous ligand, RU486, regardless of the participation of endogenous ER or its mutants. Here, we demonstrated that the protein constructed in this way could indeed effectively block the expression of ER target genes *in vitro* in cancer cell line. Its potency was tightly regulated by RU486. The effect of repressor was specific to ER target genes, since it had no effect on other nuclear receptors tested so far. The availability of this regulatable repressor provides us a powerful tool to dissect the role of ER target genes in the growth of breast cancer. The success of the regulatable repressor in shutting-off ER target genes will have far reaching effect on current hormone therapy as well as on designation of future strategy for gene therapy of breast cancer.